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Molecular controls of radiation-induced apoptosis in the neonatal rat kidney

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Molecular controls of radiation-induced apoptosis in the neonatal rat kidney. The incidence of cell death due to radiation was examined in a neonatal *in vivo* model. Differences in the induction of apoptosis, amount of cell proliferation, S-phase cell death, Bcl-2 and p-53 expression could best be explained by the differences in the zonal state of differentiation and development. The present *in vivo* model may thus be useful in testing radiation therapies for renal cancer.

Apoptosis is a gene-driven cell death process that is widely distributed under both normal and pathological conditions, and was first described by Kerr, Wyllie, and Currie [1]. In neonatal tissues such as cerebellum, muscle, and some urogenital tissues, radiation-induced changes that include apoptosis have been described in the literature, but the molecular controls are poorly defined.

The kidney of the neonatal rat is not fully developed at birth and remains so until approximately two weeks after birth when the development to the adult form is completed. There are two distinct growth zones in the immature kidneys: an outer highly proliferative nephrogenic zone that lacks adult differentiation, surrounding an inner zone of differentiated, adult-like nephron structures with rudimentary glomeruli, and having low levels of proliferation [2]. Thus, in the one tissue, comparisons may be made between molecular controls of induced apoptosis in highly proliferative, undifferentiated renal cells versus mature, differentiated cells. The results of such studies have application in many instances of renal disease, but particularly in treatment of renal cancers, where the proliferative cancer needs to be targeted by chemotherapy or radiotherapy, whereas the normal quiescent renal cells need to be protected.

The execution of renal apoptosis is under fine physiological and molecular control [3, 4]. This control is important, as it allows the cell to determine its fate according to extrinsic and intrinsic survival or death factors and, perhaps more importantly, offers an avenue for our manipulation of its incidence. Bcl-2 is an important renal apoptosis regulator [3, 5]. Abnormal kidney development and augmented metanephric apoptosis occurs in Bcl-2–deficient mice [6, 7]. In normal fetal kidney, the distribution of apoptotic cells is inversely correlated with the expression of Bcl-2. Bcl-2 deficiency had dramatic consequences in the kidney, with the development of polycystic kidneys and evidence of exfoliated renal epithelial cells in the convoluted tubules. There are also several studies that demonstrate that up-regulation of wild-type p53 modulates levels of radiation-induced apoptosis and that this increased expression is necessary for the repair of DNA strand breakage from the radiation damage to be repaired [8].

In this article, we analyze the incidence of radiation-induced apoptosis in the neonatal *in vivo* model. An associated expression of the two apoptosis-related genes, p53 and Bcl-2, was also studied. The induction of apoptosis is sometimes dependent on new macromolecular synthesis, and the role of new protein synthesis in the induction of apoptosis by irradiation was analyzed via the administration of concurrent cycloheximide (CHX), a potent protein synthesis inhibitor.

METHODS

Ethical approval was given by the University of Queensland Animal Experimentation Ethics Committee (AEEC number PATH/423/96).

Animals, tissue, and treatment

The kidneys from neonatal (4- to 5-day-old) rats ($N = 4$ per control and treated group) were studied after 5Gy of whole-body X-irradiation. Tissue was collected at 2, 4, 6, 8, and 24 hours after treatment**.** Treatment regimens were as follows: (*a*) radiation treatment alone, (*b*) 1.5 mg/kg body wt of CHX one hour before treatment (analysis of role of protein synthesis), (*c*) 5 μCi/g body weight of [6-^{3}H]-thymidine one hour before tissue collection (analysis of proliferative status of tissue), and (*d*) 5 μCi/g

Key words: Bcl-2, p53, protein synthesis, cytoheximide, cell death, renal cancer.

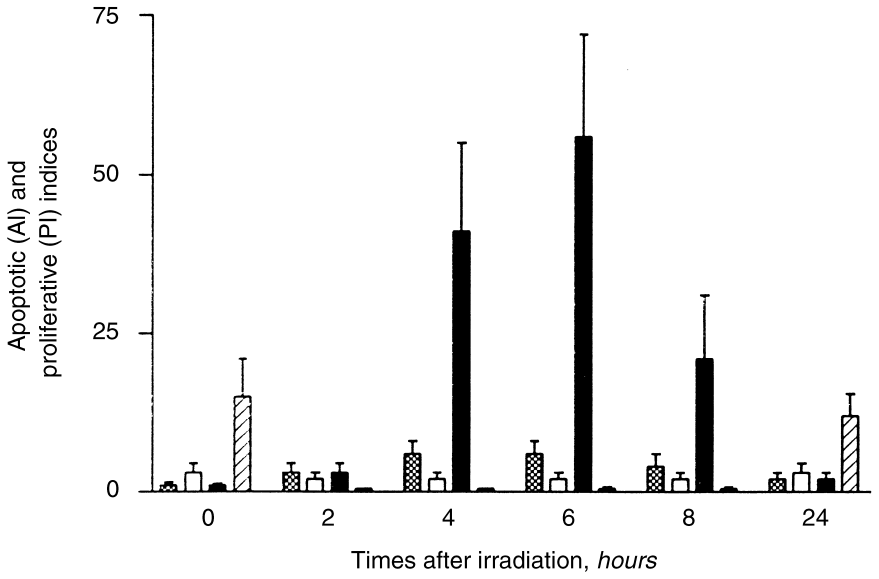

Fig. 1. Apoptotic (AI) and proliferative (PI) indices (mean ± SEM of apoptosis or labeled cells per high-power microscope field) at the set experimental times for the nephrogenic or differentiated zones. Symbols are: (▨) AI differentiated; (□) PI differentiated; (■) AI nephrogenic; (▨) PI nephrogenic.

body wt of [6-^{3}H] thymidine one hour before radiation treatment (analysis of S-phase cells undergoing apoptosis). Appropriate control groups were used.

Analyses

The following analyses were performed: (*a*) temporal and spatial incidence of radiation-induced apoptosis in the distinct nephrogenic or differentiated zones, with and without CHX-pretreatment, using light and electron microscopy [9]; (*b*) verification of presence of apoptosis by DNA gel electrophoresis; (*c*) immunolocalization of two apoptosis-related genes, p53 and Bcl-2; (*d*) autoradiography for postradiation levels of cell proliferation and for S-phase cells that have undergone apoptosis.

Statistical analyses

Data were analyzed using standard statistical methods using Student's *t*-test for unpaired samples.

RESULTS

Quantitation of apoptosis and cell proliferation

In renal sections from control animals, apoptosis was occasionally identified in the nephrogenic zone, with little evidence of it in the mature zone. Figure 1 demonstrates the apoptotic and proliferative indices (mean ± SEM of apoptosis or labeled cells per high-power microscope field) at the set experimental times for the nephrogenic or differentiated zones. After irradiation, extensive, often synchronous, apoptosis occurred in the nephrogenic zone, peaking at six hours post-irradiation and returning almost to base levels by 24-hours post-treatment. In the mature zone, apoptosis was increased over control levels after two hours, but was much lower (by a factor of approximately 10) in this zone than the nephrogenic zone. Cell proliferation was almost completely negated after irradiation in both zones and was returned to normal levels by 24 hours. Radiation-induced apoptosis in both zones was almost negated by concurrent application of CHX (not shown in this figure).

Histology

Figure 2 is from hematoxylin and eosin (HE)-stained paraffin sections of control (Fig. 2A), six-hours irradiated (Fig. 2B), and CHX-treated plus six-hours irradiated tissue (Fig. 2C). Control sections had many mitotic cells and occasional apoptotic bodies. The nephrogenic zone of irradiated kidneys showed massive, synchronous apoptosis in the S-shaped ampullae. The differentiated zone showed little increase in apoptosis. CHX pretreatment negated the induction of apoptosis, but cells were swollen and vacuolated. This latter feature may have been reversible injury.

Verification of apoptosis

The presence of apoptosis was verified using electron microscopy and DNA gel electrophoresis. The stereotypical ultrastructural changes of apoptosis are clearly seen in Figure 3A, where the cytoplasm and nucleus of the apoptotic cells are condensed and the nuclear chromatin has formed in a crescentic pattern against their nuclear envelopes. DNA gel electrophoresis (Fig. 3B) showed typical banding patterns of apoptosis.

Autoradiography

Figure 4 shows the results for ^{3}H-thymidine uptake in control or irradiated kidney. The control kidney section has high levels of label in the nephrogenic zone com-

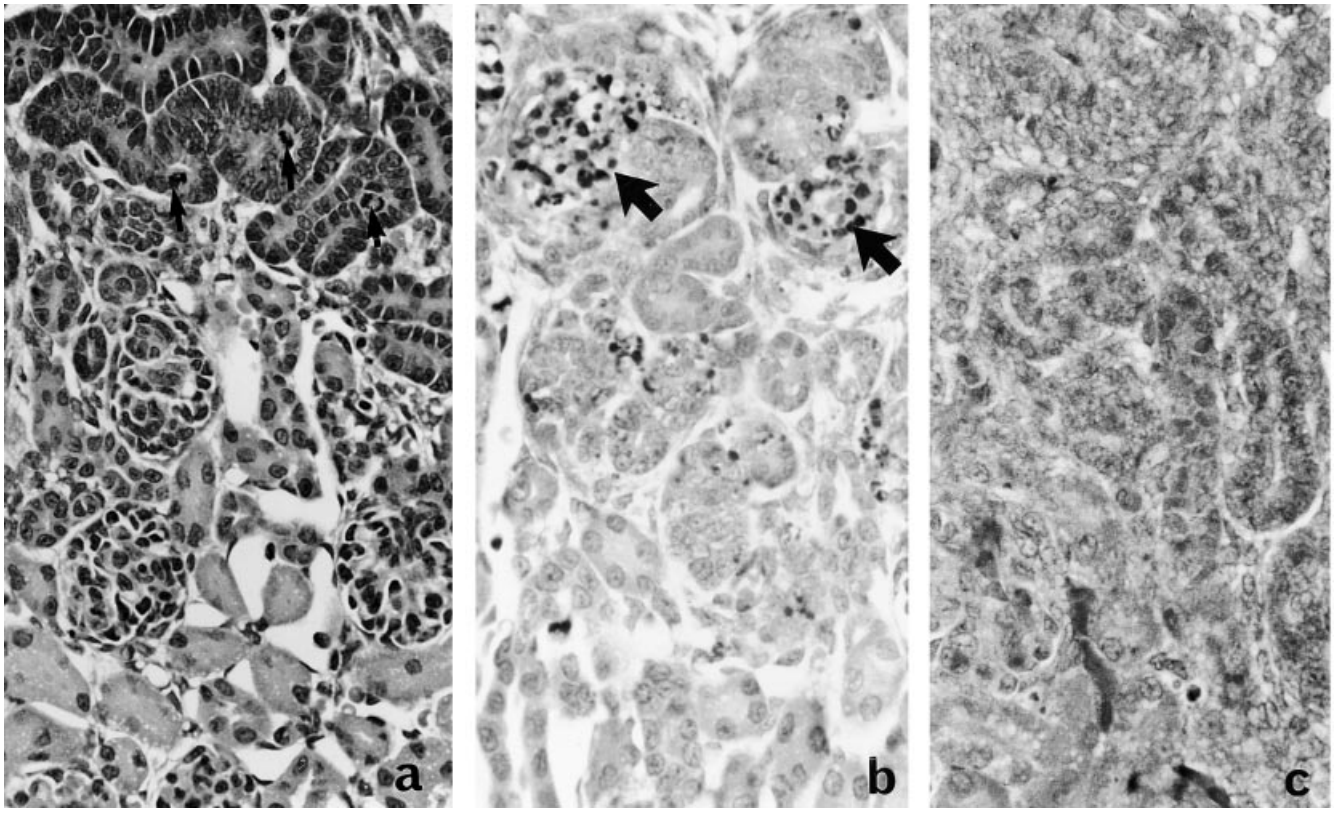

Fig. 2. Control tissue sections had many mitotic cells (small arrows) and occasional apoptotic bodies (*A*). After irradiation, the nephrogenic zone showed massive, synchronous apoptosis in the S-shaped ampullae (arrow), whereas the differentiated zone showed little increase in apoptosis (6-hr postirradiation) (*B*). Cycloheximide (CHX) treatment prior to irradiation negated the induction of apoptosis, but cells were swollen and vacuolated (*C*; ×450).

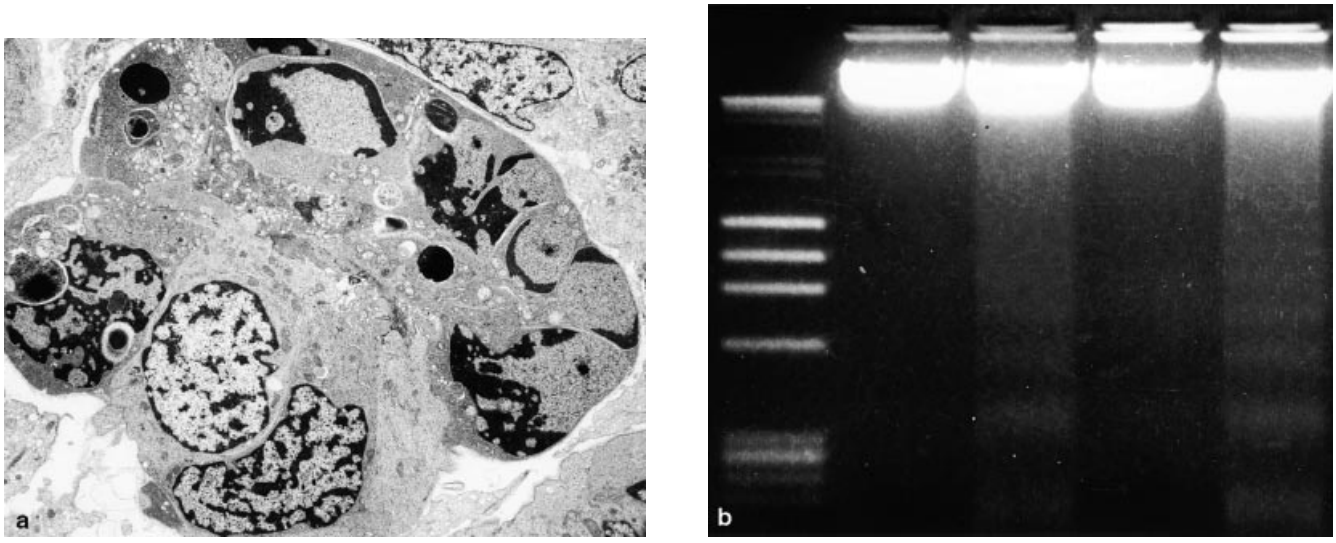

Fig. 3. Typical ultrastructural changes of apoptosis are clearly seen in (*A*), with several cells showing crescentic condensation of chromatin against the nuclear membrane and several apoptotic bodies visible. (*B*) DNA gel electrophoresis showed banding patterns of apoptosis (multiples of 180 to 200 base pairs) in lanes 3 and 5 (nephrogenic zone) compared with the relevant control tissue in lanes 2 and 4. Lane 1 is the DNA size marker.

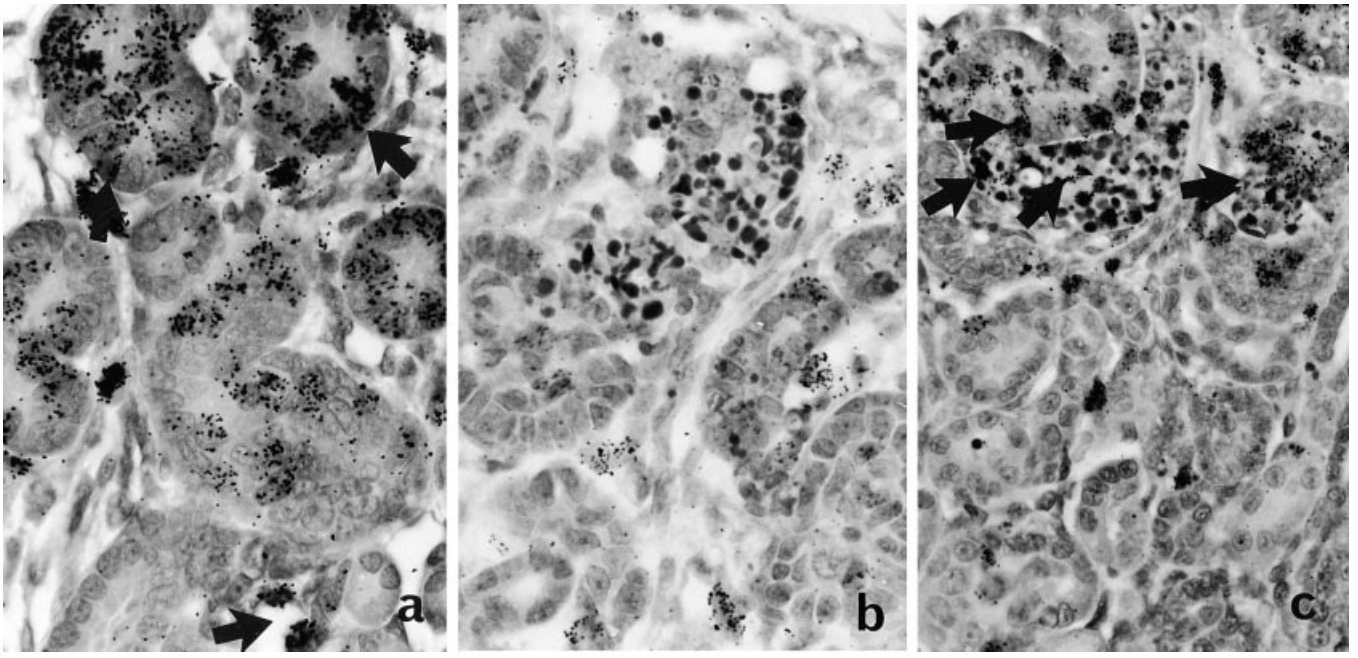

Fig. 4. Autoradiography. The control kidney section has a high level of label in the nephrogenic zone compared with moderate labeling in the differentiated zone (labeled cells arrowed; *A*). Pulse labeling one hour prior to the collection of tissue resulted in few labeled nuclei in either zone (*B*). When pulse labeling was done one hour prior to irradiation, many apoptotic cells had label, indicating S-phase death (*C*; ×600).

pared with moderate labeling in the differentiated zone (Fig. 4A). With pulse labeling after irradiation, one hour prior to collection of tissue, few labeled nuclei are found in either zone (Fig. 4B). After pulse labeling one hour prior to irradiation, approximately 35% of apoptotic cells in the nephrogenic zone carried label, indicating S-phase death, whereas less than 1% of apoptotic cells in the differentiated zone had a nuclear label.

Immunohistochemistry

Expression of Bcl-2 in control or irradiated renal tissue. The S-shaped nephrogenic tissue had low or no expression, with intervening cells having moderate expression. At six-hours postirradiation, the expression of Bcl-2 was found in some apoptotic foci, with similar levels to control tissue.

Expression of p53. Expression was moderate in both nephrogenic and mature zones and did not appear to vary significantly between renal sections from control or radiation-treated animals. CHX pretreatment was associated with very low levels of Bcl-2 or p53 expression in both tissues.

DISCUSSION

Apoptosis is a gene-driven cellular response to many types of pathological and physiological stimuli and is necessary for normal development in embryogenesis and for tissue homeostasis in the adult [3, 10]. It plays an important role in urogenital development, physiology, and pathology [3, 4]. The death response allows removal of many unwanted cells, such as may be found in a rapidly proliferating cell population, both before and after radiation injury. DNA is the critical radiation target. In normal immature tissues, there is an apoptotic removal of occasional stem cells present in excess of steady-state requirements or removal of stem cells within which minor DNA damage has occurred through damaging agents such as radiation. In each case, apoptosis may be considered to have a beneficial role for the organism. In particular, cells with damaged DNA, if allowed to proliferate, would carry with them potentially damaging mutations that would be detrimental to the adult organism. On the other hand, radiation-induced apoptosis may remove excess stem cells in neonatal tissues, and in this case, the level of cell death may later affect the quality of development in the adult organism. The outcome of cell death is well tolerated in most neonatal tissues, according to the cell type killed. In this study, by 24-hour post-treatment, the tissue appeared similar to the control tissue.

The differences detected in our study in the nephrogenic and differentiated zones [induction of apoptosis, level of proliferation, S-phase cell death (apoptosis), Bcl-2 and p53 expression] are best explained by the differences in the zonal state of differentiation and development. However, these unique differences may be used to identify important interrelationships between incidence

and molecular controls of cell death in neonatal urogenital tissues. In the control kidney, Bcl-2 expression was highest in the mature, differentiated zone, with little in the nephrogenic zone, although occasional renal ampullae had moderate levels of expression. After irradiation, Bcl-2 expression increased in the nephrogenic zone, typically between the developing nephrons, but expression was also found in some apoptotic cells. Its role as an anti-apoptosis gene may be acting in the surviving cells, and in the apoptotic cells, expression of Bcl-2 may be cell cycle related, a correlation found by Lu et al [11]. Expression of p53 was moderate to high in both nephrogenic and mature zones, and expression did not appear to vary significantly between renal sections from control or radiation-treated animals. At least in this model, its role in acting as a "guardian of the genome" [8] seems not to be overriding. Protein synthesis inhibition with CHX completely negated the induction of apoptosis in the nephrogenic zone, although occasional evidence of apoptosis was seen in the mature zone. Because many cancer chemotherapeutic drugs depend on diminished protein synthesis as an outcome, this *in vivo* model may also be useful to test cancer therapies for renal neoplasms, for example, in the planning of concurrent radiotherapy and chemotherapy to a maximum effect.

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